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Rapid PCR amplification protocols decrease the turn-around time for detection of antibiotic resistance genes in Gram-negative pathogens

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ABSTRACT

A previously designed end-point multiplex PCR assay and singleplex assays used to detect β-lactamase genes were evaluated using rapid PCR amplification methodology. Amplification times were 16–18 minutes with an overall detection time of 1.5 hours. Rapid PCR amplifications could decrease the time required to identify resistance mechanisms in Gram-negative organisms.

One of the most precious commodities in any laboratory is time. This is true for research, reference, and clinical laboratories. A big problem facing healthcare worldwide is the increasing number of multidrug resistant (MDR) Gram-negative bacterial infections. Identification of these resistance mechanisms is extremely important whether the identification is carried out in a research laboratory for a surveillance study, a reference laboratory for infection control purposes, or in a clinical laboratory to aid in determining the most appropriate antibiotic for the infected patient. β-Lactamase production is the most prevalent β-lactam resistance mechanism among Gram-negative pathogens (Hawkey and Jones, 2009). Some of the more important β-lactamases worldwide contributing to therapeutic failure include extended-spectrum β-lactamases (ESBLs); carbapenemases such as *Klebsiella pneumoniae* carbapenemases (KPCs), VIM, IMP, and NDM; and plasmid-mediated AmpC β-lactamases (pmAmpCs) (Denisuik et al., 2013; Dhillon and Clark, 2012; Hombauch et al., 2013; Mangold et al., 2011; Rand et al., 2011). In many cases, the resistance mechanisms responsible for these MDR organisms are difficult to identify using conventional phenotypic testing methodology (Denisuik et al., 2013; Rand et al., 2011). Molecular diagnostic assays using conventional end-point PCR can take 2–3 hours to complete, followed by gel electrophoresis. Real-time assays can also take up to 2 hours to complete. This time constraint can impede laboratories trying to identify organisms carrying these genes.

A method that could decrease the time for amplification and thus the turn-around time for results would be a useful tool for laboratories. Therefore, the utility of rapid PCR amplification to detect β-lactamase resistance genes in Gram-negative isolates was evaluated and compared to conventional PCR amplification protocols. Rapid amplification methodology requires a higher concentration of magnesium chloride or magnesium sulfate along with higher primer concentrations in comparison to traditional end-point PCR protocols. The purpose of this study was 2-fold: 1) to optimize previously designed primer sets that amplify prevalent β-lactamase genes using singleplex PCR and 2) to adapt a previously designed and optimized end-point AmpC multiplex PCR assay for pmAmpC detection using rapid cycling parameters.

Previously characterized clinical isolates known to carry various Class A, B, and D β-lactamase genes were used as controls to optimize singleplex PCR amplification conditions. Primer sets used in singleplex PCRs can serve as a starting point for the detection of key β-lactamase genes, especially in research laboratories as demonstrated in this study. We adapted existing primers sets already tested for specificity in our laboratory using conventional end-point PCR for use in a rapid amplification protocol. The thermal cycler used in these experiments was the Philisa® Thermal Cycler (Streck, Inc., Omaha, NE, USA). DNA template was obtained by heating bacterial cells to 100 °C and using 2 μL of supernatant per 50-μL reaction. The Philisa® Thermal Cycler is capable of using 10–50 μL PCR reactions following modifications to the PCR master mix components. PCR reactions were prepared in thin-walled, elongated tubes crafted for the Philisa® Thermal Cycler, which allow for efficient heat transfer. Primer sets previously used on conventional and real-time thermal cyclers were optimized for rapid cycling conditions and included primers for Class A (KPC-like, CTX-M Groups 1 and 9, TEM-like, and SHV-like), Class B (NDM-like, SPM-like, GIM-like, IMP-like, and VIM-like), and Class D (OXA-1-like, OXA-9, OXA-23-like, OXA-24-like, OXA-40-like, and OXA-58-like) β-lactamase genes (Table 1). Amplicon sizes ranged

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from 106–750 bp. PCR products generated using these rapid cycling conditions were electrophoresed, stained with ethidium bromide, and imaged using Kodak computer software. PCR products using primer sets in Table 1 are shown in Figs. 1 and 2.

Singleplex PCR amplifications using this methodology were completed within 16–18 minutes for all primer sets tested using either *Taq* or KOD polymerases (Fermentas, ThermoFisher and Novagen, Millipore, respectively). Programmed conditions for all singleplexes included an initial denaturation at 95 °C for 15 seconds followed by 3-step cycling conditions for 30 cycles; 95 °C denaturation for 5 seconds, annealing for 5 seconds, and 72 °C/70 °C extension for 15 seconds using either *Taq* or KOD polymerases, respectively. Magnesium concentration and annealing temperatures were dependent upon the primer set tested and ranged from 4.0 mmol/L to 5.0 mmol/L and 49 °C to 62 °C, respectively (Table 1). Each PCR reaction consisted of 1X *Taq*+KCl PCR buffer, 4.0 mmol/L or 5.0 mmol/L $MgCl_2$, 200 μmol/L dNTP mix, 2 μmol/L of forward and reverse primers, and 1 U of Fermentas *Taq* (5 U/μL). When using the KOD polymerase, the PCR reaction included 1X KOD HotStart buffer, 4.0 mmol/L or 5.0 mmol/L $MgSO_4$, 200 μmol/L dNTP mix, 3 μmol/L of forward and reverse primers, and 1 U of KOD HotStart DNA polymerase (1 U/μL). The decrease in cycling time reduced the overall time of detection

Table 1
Primers used for the detection of common β-lactamase genes using singleplex PCR.

Target	Primer name	Sequence (5′ to 3′)	Expected amplicon size (bp)	$MgCl_2$ concentration (mmol/L)	Additional targets	Nucleotide position	GenBank accession number[a]
Class A β-lactamases							
KPC	KPC-F2	GTATCGCCGTCTAGTTCTG	209	4.0	KPC-2-16	1235-1253	JN048641
	KPC-R6	CCTTGAATGAGCTGCACAGTGG				1443-1422	
CTX-M Group 1	CTXM-1F3	GACGATGTCACTGGCTGAGCTTAGC	499	5.0	CTX-M-1, 3, 15, 30, 32, 38, 52, 55, 57, 79, 116, 123, 136, 168	1789-1813	AY044436
	CTXM-1R2	AGCCGCCGACGCTAATACA				2287-2269	
CTX-M Group 9	CTXM-914F	GCTGGAGAAAAGCAGCGGAG	474	5.0	CTX-M-9, 14, 19, 24, 27, 65, 93, 98, 104–106, 110–113, 121–123, 125,	1857-1876	AF252622
	CTXM-914R	GTAAGCTGACGCAACGTCTG			126, 129, 130, 134,	2330-2311	
TEM	TEM-prime1F	AGATCAGTTGGGTGCACGAG	750	4.0	Universal	183-202	JX129214
	TEM-prime2R	TGCTTAATCAGTGAGGCACC				932-913	
SHV	SHV-prime2F	GGGAAACGGAACTGAATGAG	149	4.0	Universal	482-501	JN676879
	SHV-prime3R	ATCGTCCACCATCCACTGCA				630-611	
Class B β-lactamases							
NDM	NDM1-F1	GCACACTTCCTATCTCGACATGC	209	4.0	NDM-1-4, 6	180-202	JQ734687
	NDM1-R1	CCATACCGCCCATCTTGTCC				388-369	
VIM	VIM1F	GGTGTTTGGTCGCATATCGC	504	4.0	VIM-1-4,11, 19, 20, 23–25, 27, 28, 31, 33-38	154-173	JN566054
	VIM1R	CCATTCAGCCAGATCGGCATC				657-637	
IMP	IMP1F	GGAATAGAGTGGCTTAATTC	328	4.0	IMP-1, 2, 4, 6–8, 10, 15, 19, 22, 25, 26, 32, 37, 38, 40-42	307-326	EU368856
	IMP1R	CAACCAGTTTTGCTTTACC				634-616	
SPM	SPM-RTF1	CCCATCCTGTTCCAGCGG	197	4.0	SPM-1	2775-2792	AY341249
	SPM-RTR1	GGCATCTCCCAGATAACC				2971-2954	
GIM	GIM-RTF1	GCCCGTGAAGGAAAGCCG	106	4.0	GIM-1	1410-1427	AJ620678
	GIM-RTR1	CCTCTGTATGCCCAGCACC				1515-1497	
Class D β-lactamases							
OXA-9	OXA91F	CGTCGCTCACCATATCTCCC	315	4.0	OXA-9	4498-4517	AF034958
	OXA91R	CCTCTCGTGCTTTAGACCCG				4812-4793	
OXA-1 Group	OXA1F	TGTGCAACGCAAATGGCAC	579	4.0	OXA-1, 4, 30, 31	400-418	JN003856
	OXA1B14	CGACCCCAAGTTTCCTGTAAGTG				978-956	
OXA-23 Group	OXA23-intF1	CAGAATATGTGCCAGCCTC	546	4.0	OXA-23, 27, 49, 73, 133, 134, 165–171, 225, 239	1451-1469	JN207493
	OXA23-intR1	GCATTTCTGACCGCATTTCC				1996-1977	
OXA-24 Group	OXA24-intF1	CACCTATGGTAATGCTCTTGC	501	4.0	OXA-24-26, 40, 72, 139, 207	4326-4346	JN207494
	OXA24-intR1	CAACCTACCTGTGGAGTAACAC				4826-4805	
OXA-40 Group	OXA40-intF1	CACCTATGGTAATGCTCTTGC	491	4.0	OXA-24-26, 33, 40, 72, 139, 207	192-212	AF509241
	OXA40-intR1	GTGGAGTAACACCCATTCC				682-664	
OXA-58 Group	OXA58-intF1	GTGGGATGGAAAGCCACG	376	4.0	OXA-58, 96, 156	1813-1830	JQ412186
	OXA58-intR1	CACTTGCGGGTCTACAGC				2188-2171	

[a] Sequence used for primer design.

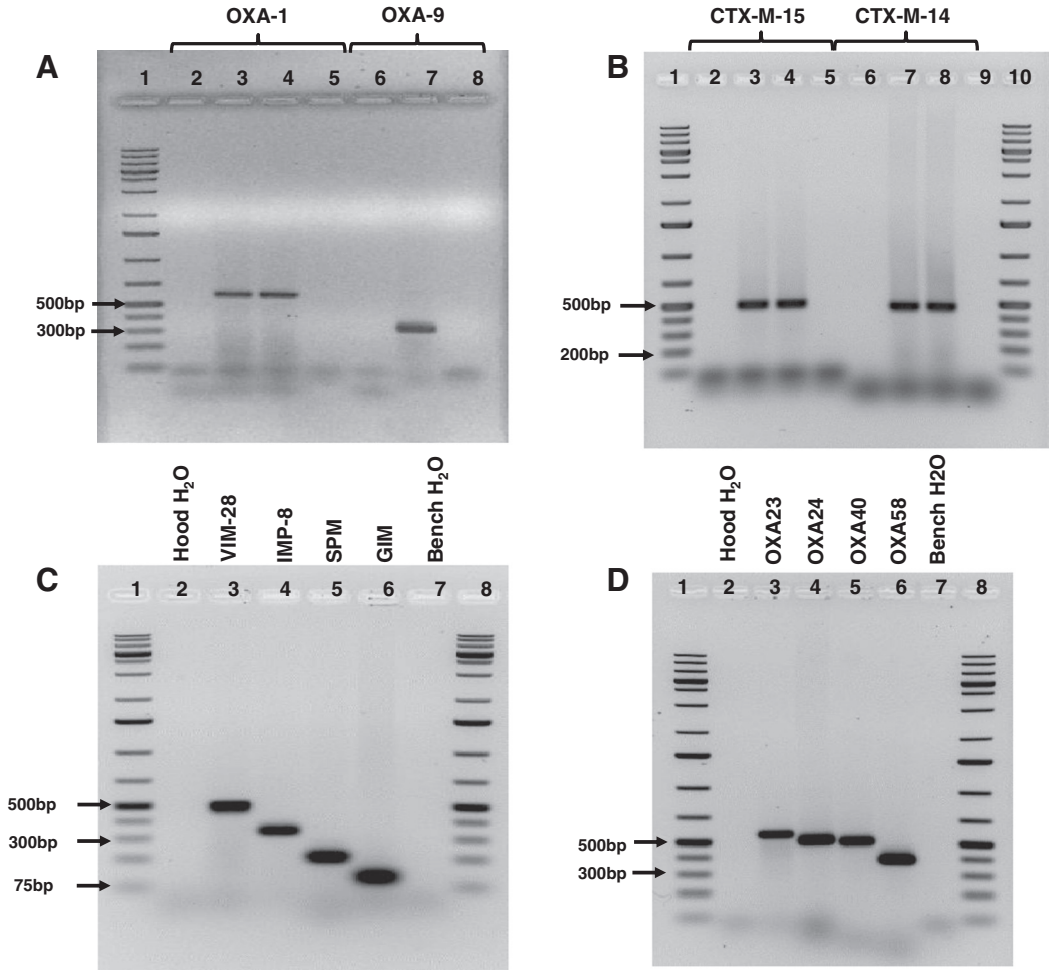

Fig. 1. Agarose gel electrophoresis of singleplex PCRs using rapid amplification methodology and *Taq* DNA polymerase. Panel A. Singleplex PCR amplifications detecting OXA-1 and OXA-9 templates. Lane 1: GeneRuler 1 kb Plus ladder (Fermentas). Lanes 2, 5, 6, 8: no template controls. Lanes 3 and 4: OXA-1 template. Lane 7: OXA-9 template. Panel B. Singleplex PCR amplification detecting CTX-M-15 and CTX-M-14 templates. Lanes 1 and 10: GeneRuler 1 kb Plus ladder. Lanes 2, 5, 6, and 9: no template controls. Lanes 3 and 4: CTX-M-15 template. Lanes 7 and 8: CTX-M-14 template. Panel C. Singleplex PCR amplifications detecting VIM, IMP, SPM, and GIM templates. Lanes 1 and 8: GeneRuler 1 kb Plus ladder. Lanes 2 and 7: no template controls. Lanes 3, 4, 5, and 6: VIM-28, IMP-8, SPM, and GIM templates, respectively. Panel D. Singleplex PCR amplifications detecting OXA-23, OXA-24, OXA-40, and OXA-58 templates. Lanes 1 and 8: GeneRuler 1 kb Plus ladder. Lanes 2 and 7: no template controls. Lanes 3, 4, 5, 6: OXA-23, OXA-24, OXA-40, and OXA-58, respectively. Agarose concentrations for all gels was 1.5%.

from 5 hours to 1.5 hours. Using *Taq* polymerase rather than KOD decreased the total cost of the singleplex PCRs.

Primer sets previously designed for the detection of bla_{CMY-2}, bla_{FOX}, bla_{ACT}, bla_{ACC}, bla_{MOX}, and bla_{DHA} were used in adapting the *ampC* multiplex PCR assay for rapid amplification end-point PCR (Table 2) (Perez-Perez and Hanson, 2002). However, the CMY-2 primer set and the forward primer specific for FOX were redesigned for optimization of this assay. The CMY-2 primer set was redesigned to prevent cross hybridization with the chromosomal *ampC* gene in *Escherichia coli*, which had not been observed for this primer set in the original *ampC* multiplex PCR assay. The new CMY-2 primer set produced an amplicon that was similar in size to the original FOX primer set. Therefore, the forward FOX primer was redesigned to generate a slightly larger amplicon of 247 bp instead of 190 bp. Amplicon sizes ranged from 106 bp to 520 bp. *E. coli* transformants containing pmAmpC gene fragments served as positive controls. DNA template was isolated using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Two microliters (~50 ng) of diluted template was used in a 50-μL reaction.

The optimized *ampC* multiplex PCR conditions adapted for rapid PCR amplification included an initial denaturation at 95 °C for 15 seconds followed by 3-step cycling for 30 cycles; denaturation at 95 °C for 5 seconds, annealing at 65 °C for 10 seconds, and extension at 70 °C for 15 seconds. A final extension step was included at 70 °C for 15 seconds. The extension temperature for the KOD polymerase was 70 °C, while *Taq* polymerase had an extension temperature of 72 °C extension. The thermal cycler used for these amplifications was the Philisa® Thermal Cycler. The annealing temperature for the *ampC* multiplex assay was increased by 1 °C for adaptation on the Philisa®. Each 50-μL PCR reaction contained 1X KOD HotStart buffer, 4.0 mmol/L $MgSO_4$, 0.1 mmol/L of each dNTP, 0.5 μL KOD HotStart DNA polymerase (1 U/μL), and 1.1 μmol/L MOX MF/MR; 0.5 μmol/L CMY2 MF5/MR2; 0.8 μmol/L DHA MF/MR; and 0.3 μmol/L ACC MF/MR, EBC MF/MR, and FOX CF2/MR primers. Each amplification reaction shown in Fig. 3 contained all 6 primer sets and DNA template from their respective AmpC family. One major amplified product was shown for each AmpC control. Amplification using rapid cycling parameters was completed in 18 minutes compared to 2.5 hours with end-point PCR amplification protocols. PCR products were visualized as described above.

Rapid amplification is an efficient tool that can be used for multiple applications in research, reference, and clinical laboratories. Here, we used existing primer pairs for the detection of β-lactamase genes. Applications already in use in any research, reference, or diagnostic laboratory could be easily converted for use in rapid

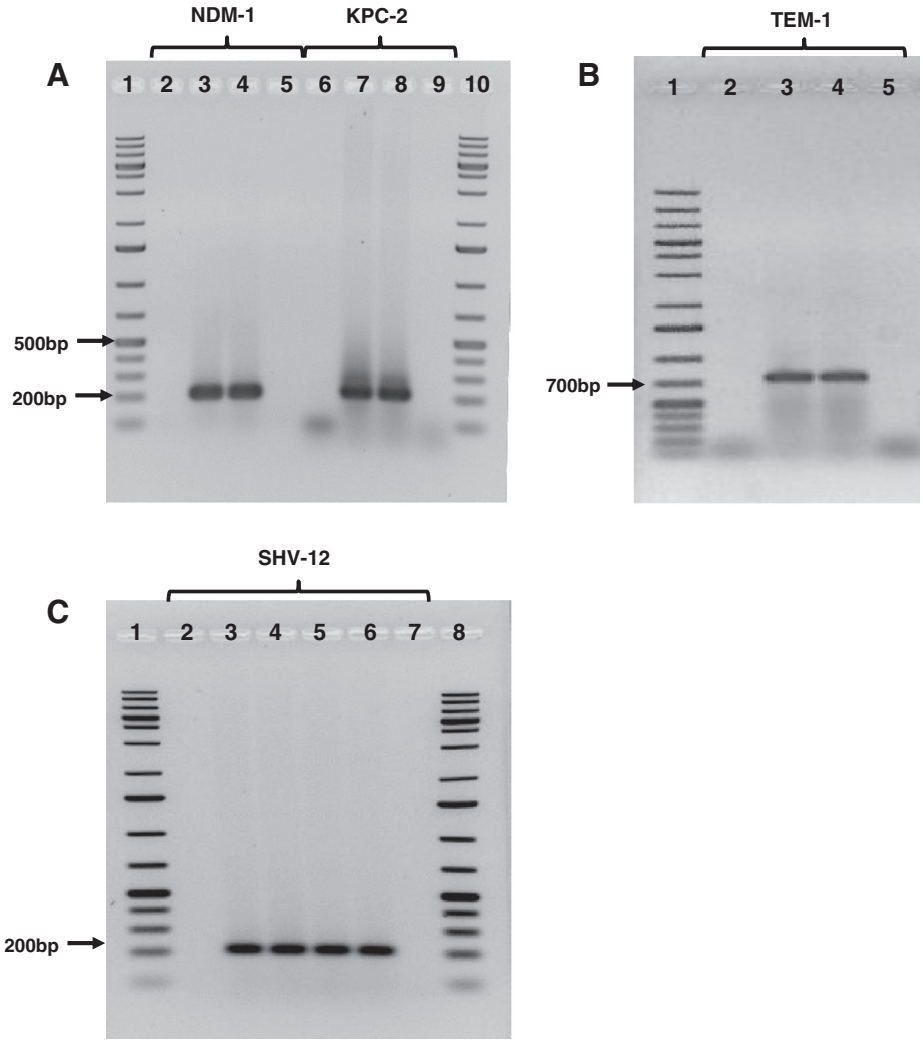

Fig. 2. Agarose gel electrophoresis of singleplex PCRs for NDM, KPC, TEM, and SHV genes using rapid amplification methodology and *Taq* DNA polymerase. Panel A. Singleplex PCR amplifications detecting NDM-1 and KPC-2 templates. Lanes 1 and 10: GeneRuler 1 kb Plus ladder. Lanes 2, 5, 6, 9: no template controls. Lanes 3 and 4: NDM-1 templates. Lanes 7 and 8: KPC-2 templates. Panel B. Singleplex PCR amplification detecting TEM-1 template. Lane 1: GeneRuler 1 kb Plus ladder. Lanes 2 and 5: no template controls. Lanes 3 and 4: TEM template. Panel C. Singleplex PCR detecting SHV-12 template. Lanes 1 and 8: GeneRuler 1 kb Plus ladder. Lanes 2 and 7: no template controls. Lanes 3, 4, 5, 6: SHV-12 template. Agarose concentrations for all gels was 1.5%.

amplification protocols. The versatility of using either *Taq* or a proofreading enzyme allows for downstream applications such as subcloning or sequencing of products without re-optimization of the protocol. Rapid amplification methodology has the potential to enhance the speed by which laboratories can detect resistance genes in any microbial organism.

Table 2
Primers used for AmpC multiplex PCR adaptation.

Primer name	Sequence (5′ to 3′)	Target (s)	Expected amplicon size (bp)	Nucleotide position	GenBank accession number[a]
MOX MF	GCTGCTCAAGGAGCACAGGAT	MOX-1-7, CMY-1, 8–11, 19	520	358-378	D13304
MOX MR	CACATTGACATAGGTGTGGTGC			877-856	
DHA MF	AACTTTCACAGGTGTGCTGGGT	DHA-1, 5, 6, 7	405	1244-1265	Y16410
DHA MR	CCGTACGCATACTGGCTTTGC			1648-1628	
ACC MF	AACAGCCTCAGCAGCCGGTTA	ACC-1, 2, 4	346	861-881	AJ133121
ACC MR	TTCGCCGCAATCATCCCTAGC			1206-1186	
EBC MF	TCGGTAAAGCCGATGTTGCGG	ACT-1, 2, 5, 8, 14, 15, 16 MIR-1, 2, 3, 4, 6	302	1115-1135	M37839
EBC MR	CTTCCACTGCGGCTGCCAGTT			1416-1396	
FOX CF2	GCCGAGGCTTACGGGATCAAG	FOX-1-9	247	1418-1438	X77455
FOX MR	CAAAGCGCGTAACCGGATTGG			1664-1644	
CMY2 MF5	CCGAAGCCTATGGCGTGAAATCC	CMY-2, 4, 12, 14–16, 25, 27, 29, 30, 32, 33, 38, 39, 42, 44–51, 53–56, 58–68, 71–73, 75–77, 79–81, 84, 87	106	3074-3096	JQ318857
CMY2 MR2	GCAATGCCCTGCTGGAGCG			3179-3161	

[a] Sequence used for primer design.

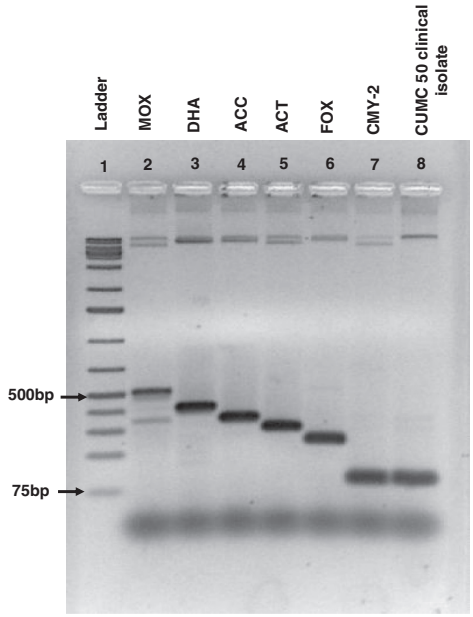

Fig. 3. Agarose gel electrophoresis of *ampC* multiplex PCR using rapid amplification methodology and KOD HotStart DNA polymerase. AmpC multiplex PCR showing amplification of 6 *E. coli* transformant controls and 1 positive clinical isolate. Separation of amplicons was completed using a 2% agarose gel. Lane 1: GeneRuler 1 kb Plus ladder. Lanes 2–7 are the *E. coli* transformant controls arranged in decreasing size: MOX (520 bp), DHA (405 bp), ACC (346 bp), ACT (302 bp), FOX (247 bp), and CMY-2 (106 bp). Lane 8 represents the DNA from a clinical isolate positive for CMY-2.

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